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Role of nuclear factor-κB (NFκB) in microglial polarization in correlation with neuroinflammatory mechanism at the hippocampal cornu ammonis (CA) 1 region after acute and chronic phase of global ischemic brain injury in rats

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ABSTRACT

Ischemic brain injuries can result in hippocampal injury due to its vulnerability to ischemia, specifically the CA1 region. Ischemic injury to this region alters nerve cells, synapses, and non-neural hippocampal tissue and causes hippocampal sclerosis. This injury could be mediated by microglia via the neuroinflammation pathway. However, the neuroinflammatory mechanism underlying hippocampal ischemic injury is still unclear. This study aimed to investigate the role of NF-κB in microglia polarization which affects the hippocampal area after ischemic injury. We conducted a quasi-experimental study, using 24 male Sprague Dawley rats aged 4 wk old and weighing 100 g. The rats were grouped into 4 different groups (CL1 as acute, CL3 as subacute, CL7 as chronic, and SO as control groups) and performed bilateral common carotid artery ligation to induce global ischemic injury in the brain. The difference in microglial activation was tested using immunohistochemistry for CD68. Moreover, polymerase chain reaction (PCR) was utilized to assess mRNA expression differences in IL1β, IL6, TNFα, and NF-κB. An increase in the number of positive CD68 fraction areas in CL1, CL3, and CL7 compared to the SO group (p=0.002) was shown after bilateral common carotid artery ligation. Such ligation also induced a significantly higher mRNA expression of IL1β (p=0.004), IL6 (p=0.028), TNFα (p=0.028), and NF-κB (p=0.002) in the CL1, CL3, and CL7 groups, compared to the SO group. In conclusion, NF-κB is the key player in hippocampal injury in the CA1 region following ischemic event by differentiating microglia into M1 phenotype form and initiates the neuroinflammatory cascade via IL1β, IL6, and TNFα in all phases.

ABSTRAK

Cedera iskemik otak dapat mengakibatkan kerusakan hipokampus, khususnya regio CA1 yang rentan terhadap kejadian iskemia. Cedera iskemik pada regio ini dapat mengubah sel saraf, sinaps, serta jaringan hipokampus lainnya dan menjadi penyebab sklerosis hipokampus. Cedera iskemik tersebut dapat diperantarai oleh mikroglia melalui jalur neuroinflamasi. Namun demikian, mekanisme neuroinflamasi yang mendasari cedera iskemik hipokampus masih belum diketahui dengan jelas. Dalam penelitian ini, penulis melakukan investigasi tentang peran NF-κB pada proses polarisasi mikroglia yang diketahui memengaruhi area hipokampus paska cedera iskemia. Penulis melakukan studi kuasi eksperimental menggunakan 24 tikus Sprague Dawley berumur 4 minggu dan berat 100 gr untuk kemudian dimasukkan ke dalam 4 kelompok (CL1 sebagai model iskemi akut, CL3 sebagai model iskemi subakut, CL7 sebagai model iskemia kronik, dan SO sebagai kontrol) dan melakukan ligasi bilateral arteri karotis komunis untuk menginduksi cedera iskemia global pada otak. Aktivasi mikroglia diperiksa dengan pemeriksaan imunohistokimia CD68, sedangkan PCR digunakan untuk melihat ekspresi IL1β, IL6, TNFα and NF-κB. Ligasi bilateral arteri karotis komunis meningkatkan jumlah fraksi area positif CD68 pada kelompok CL1, CL3, dan CL7 dibandingkan SO. Ligasi tersebut juga meningkatkan ekspresi IL1β (p=0.004), IL6 (p=0.028), TNFα (p=0.028) dan NF-κB (p=0.002) pada CL1, CL3, dan CL7 dibandingkan SO. Dapat disimpulkan bahwa NF-κB merupakan kunci utama dalam proses cedera hipokampus pasca cedera iskemik dengan mendiferensiasi mikroglia menjadi bentuk fenotip M1 dan menginisiasi neuroinflamasi melalui IL1β, IL6, dan TNFα pada seluruh fase.

Keywords:
NF-κB;
microglial polarization;
ischemic brain injury;
M1;
bilateral carotid ligation

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INTRODUCTION

Ischemic brain injuries are a common occurrence that results in a high rate of mortality and morbidity which impair a patient's life.[1] Approximately 17 million people suffer from ischemic brain injury annually.[2] According to a study conducted by the World Stroke Organization, 5.5 million people suffered from stroke-related death in 2019.[3] This condition demonstrates the significant burden on patients, families, and healthcare systems.

Neuroinflammation plays an essential role following ischemic brain injury. It is capable of destroying, protecting, and repairing brain tissue.[4] Ischemia-induced inflammation can manifest itself in two ways: activation of resident cells and infiltration of peripheral inflammatory cells into the brain parenchyma.[5] The release and production of reactive oxygen species (ROS), cytokines, and chemokines regulate neuroinflammatory mechanisms.[6] Additionally, innate immune cells, myeloid cell infiltration, and adaptive immune cells also occurred during the inflammatory process.[7]

Among these innate cells, microglia are considered important in holding a significant role in post-ischemic neuroinflammation conditions.[8] It is referred to as a resident macrophage which provides an initial response to brain tissue damage. During inflammation, activated microglia perform the same functions as macrophages, including phagocytosis and the production of cytokines and matrix metalloproteinases (MMPs).[9-11]

Microglia that have been activated can adopt one of two phenotypes, M1 or M2. This transition between M1 and M2 is referred to as polarization.[12] M1 is also referred to as the classic type. This phenotype is proinflammatory, secreting cytokines such as IL1β, IL6, and TNFα and inducing the production of reactive oxygen and nitrogen species (ROS and nitric oxide), which can damage the nerve and glial cells.[13] Alternative-activation type is a term that refers to the M2 phenotype. It secretes anti-inflammatory factors such as IL4, IL10, and TNFβ. It aids in the survival of neurons.[14]

Polarization of activated microglia is a dynamic process. The transcription nuclear factor–κB (NF-κB) is thought to play a significant role in this process. NF-κB can activate the M1 phenotype of microglia (i.e. acting as a key transcription factor of M1 macrophages) and mediate inflammatory responses.[15] NF-κB is needed to induce a vast number of inflammatory genes (e.g. genes that encode TNFα, IL1β, IL6, and cyclooxygenase-2).[15]

The hippocampus is known to be the structure most vulnerable to injury during ischemic events, particularly in the CA1 region.[16] Ischemic injury to the hippocampus alters nerve cells, synapses, and non-neural hippocampal tissue.[16] This ischemic injury, mediated by the neuroinflammation pathway, is one of the causes of hippocampal sclerosis, a progressive loss of nerve cells in the hippocampus.[17] Hippocampal sclerosis is one of the main causes of temporal lobe epilepsy which has a good outcome if treated well. However, the underlying mechanism for this condition remains unknown.

To date, very few studies have examined the neuroinflammatory mechanism underlying hippocampal ischemic injury, specifically the role of NF-κB in microglia polarization, which may result in hippocampal damage. This is critical because it can shed light on

the mechanism of ischemic injury to the hippocampus that results in sclerosis. We hypothesized that ischemic conditions would increase NF-κB expression in the hippocampus, polarise microglia to the M1 phenotype, and increase the number of proinflammatory mediators in the hippocampus, activating the inflammation pathway and ultimately resulting in hippocampal damage. To test this hypothesis, a model of global cerebral ischemia was used in rats (i.e. bilateral CCA ligation). Immunohistochemistry was used to assess activated microglia. The expression of NF-κB and proinflammatory mediators produced in response to the ischemic event was determined using RT-PCR and electrophoresis.

MATERIAL AND METHODS

Animals

All procedures were performed by the NIH Guidelines on the care and use of vertebrate animals and approved by the Medical and Health Research Ethics Committee at Universitas Gadjah Mada, Yogyakarta, Indonesia with expediency number KE/FK/0222/EC/2021. The animals used were male Sprague Dawley rats, aged 4 wk. old and weighing 100 g. The animals were randomly assigned and housed in standard laboratory conditions, a 12-hr light-dark cycle, with controlled temperature and humidity and access to food and water ad libitum. In this study, according to Federer's formula, the sample size in each group was 6 rats. With 4 groups, a total of 24 rats were used.

Bilateral common carotid artery ligation

Four groups of six rats each were used as animal subjects: control/sham operation (SO), carotid ligation 1 (CL1), carotid ligation 3 (CL3), and carotid ligation 7 (CL7). As a control, a sham operation was used; CL1 was used as an acute model; CL3 was used as a subacute model; and CL7 was used as a chronic model. The experiments were conducted while the subjects were sedated. The anesthetic agent was pentobarbital solution 1:10 (0.1mg/10g BW) administered intraperitoneally. In the ligation model, we clamped both right and left Common Carotid Artery (CCA) for 30 min using a non-traumatic vascular clamp (Karl Hammacher GmbH, Solingen, Germany). The clamp was then released after 30 min. The surgical site was then closed with surgical thread silk 3/0 (OneMed-Healthcare, Surabaya, Indonesia). In the SO group, a cervical incision was made followed by skin closure with no artery clamping performed. On day 7, rats in the SO group were terminated. Bilateral CCA clamping was also performed in the CL1, CL3, and CL7 groups, and subjects were terminated on days 1, 3, and 7, respectively.

Brain processing

All animals were first anesthetized and decapitated after being perfused with 4% paraformaldehyde (PAM) in 50 mM phosphate buffer via transcardial approach. The sample of the brains were preserved in 4% PAM. Rat brains were

rapidly frozen in powdered dry ice and performed sectioning at 30 µm with a cryostat microtome, with six serial sets of sections collected from each brain at an interval of 180 µm for each serial set. The sections were collected in multi-well reservoirs containing a solution of 30% ethylene glycol and 30% glycerol in phosphate buffer (1M, pH 7.40). Rat brains were paraffinized and sectioned at a 4 µm interval using a microtome (Leica Biosystems, UK).

Immunohistochemical staining of CD68

The hippocampus was embedded in paraffin blocks and 4 µm sections were cut, deparaffinized, and rehydrated with 100%, 90%, 80%, and 70% alcohol, followed by heating in citrate buffer (pH 6) for antigen retrieval and blocking endogenous peroxidase with 3% H_2O_2 in PBS solution. The slides were then incubated with Background Sniper, rabbit 1st monoclonal antibody CD68 at a dilution of 1:200 (Abcam, ab32570, Cambridge, United Kingdom), TrekAvidin-HRP, rabbit 2nd monoclonal antibody Trekkie Universal Link at a dilution of 1:200 (Biocare Medical, STUHRP700, California, United States), and diaminobenzidine tetrahydrochloride (Biocare Medical, STUHRP700H L10).

Microscopic analysis

The results were viewed under a light microscope (Olympus CX22) and visualized using the Optilab software at a magnification of 400x. CA1 was chosen as the Region Of Interest (ROI). ImageJ (NIH, Bethesda, MD) was then used to subtract light background. The observer set an optimal level of threshold which would only include the area of CD68 staining in the measurement. The CD68 immunopositivity was presented as a fraction area percentage, which is the brown-stained area of the ROI.

Reverse transcriptase PCR analysis.

RNA extraction and cDNA synthesis

Total RNA was extracted using Genezol (Geneaid GZR100, Geneaid Biotech Ltd, New Taipei City, Taiwan), followed by spectrophotometric quantification of the RNA concentration. For cDNA synthesis, we used 3,000 ng RNA. Rever Tra Ace® (Toyobo Cat. No. TRT-101, Osaka, Japan) and random primer (Toyobo Cat. No. 3801) were used to generate cDNA, with the following PCR conditions: 30°C for 10 min (denaturation), 42°C for 60 min (annealing), and 99°C for 5 min (extension).

Reverse transcriptase PCR and electrophoresis.

A reverse transcriptase polymerase chain reaction (PCR) was used to amplify the following specific cDNAs: IL1β (F: AATCTGTACCTGTCCTGCGTGTT and R: TGGGTAATTTTTGGGATCTACACTCT); NF-κB (F: CACTCTCTTTTTGGAGGT and R: TGGATATAAGGCTTTACG); TNFα (F: TCCCAACAAGGAGGAGAAGT and R: TGGTATGAAGTGGCAAATCG); IL6 (F: TTGGATGGTCTTGGTCCTTAGCC and R: TCCTACCCCAACTTCCAATGCTC); GAPDH (F: GGCACAGTCAAGGCTGAGAATG and R: TCTCGCTCCTGGAAGATGGTGA). The reverse transcriptase PCR was performed using 2 µL of cDNA, 12.5 µL of Taq Master Mix (Bioron, Germany, Cat. No. S101705), 0.6 µL of forward and reverse primers, and 9.3 µL of PCR water.

For 35 cycles, the cDNA was amplified at the following temperatures: 94°C for 2 sec (initial denaturation), 94°C for 10 sec (denaturation), 60°C for 20 sec (annealing), 72°C for 1 min (extension), and 72°C for 10 min (final extension). The PCR products and a 100 bp DNA ladder were analyzed in a 2% agarose gel (Bioron Cat. No. 306009, Germany). Densitometry analysis with the ImageJ

software was used to quantify the gene's expression. The housekeeping gene GAPDH was used.

Statistical analyses

For all statistical tests, SPSS 23 software was used. Statistical analysis of mean differences between groups was performed by using one-way ANOVA, followed by LSD post-hoc analysis. Pearson correlation test was used to analyze the correlation between the results of CD68 immunohistochemistry and the PCR results of NF-κB. P value <0.05 was accepted as the level of significance.

RESULTS

Microscopy analysis

In this study, we assessed the immunopositivity of CD68 to represent the microglia activation. The immunopositivity was seen as a brown-stained area in the CA1 region, presented as a percentage of the fraction area. There were significant increases in the number of positive CD68 fraction areas in CL1, CL3, and CL7 compared to the SO group (p=0.002) (FIGURE 1). It was also found that the shape of the microglia has turned from a ramified shape in the acute phase to an amoeboid shape in the sub-acute and chronic phases. This condition was associated with the more active form of microglia in the latter phase (FIGURE 2.)

RT-PCR analysis

A significantly higher mRNA expression of IL1β, IL6, TNFα, and NF-κB was observed in the CL1, CL3, and CL7 groups, compared to the SO group (FIGURE 3). This suggests that ischemia, either in acute or chronic conditions, leads to increasing IL1β (p=0.004), IL6 (p=0.028), TNFα (p=0.028), and NF-κB production (p=0.002).

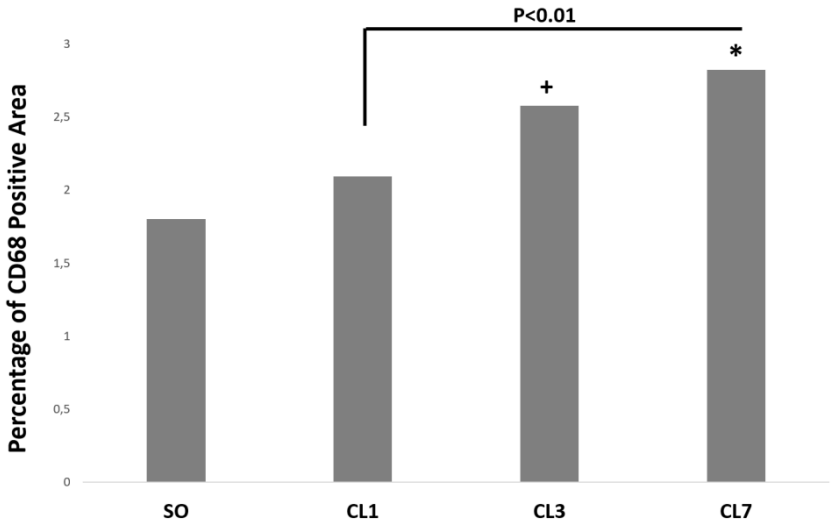

FIGURE 1. Area Fraction of IHC staining using anti-CD68. There were significant increases from the acute phase to the chronic phase. ⁺: p<0.01 *: p<0.001 compared to SO.

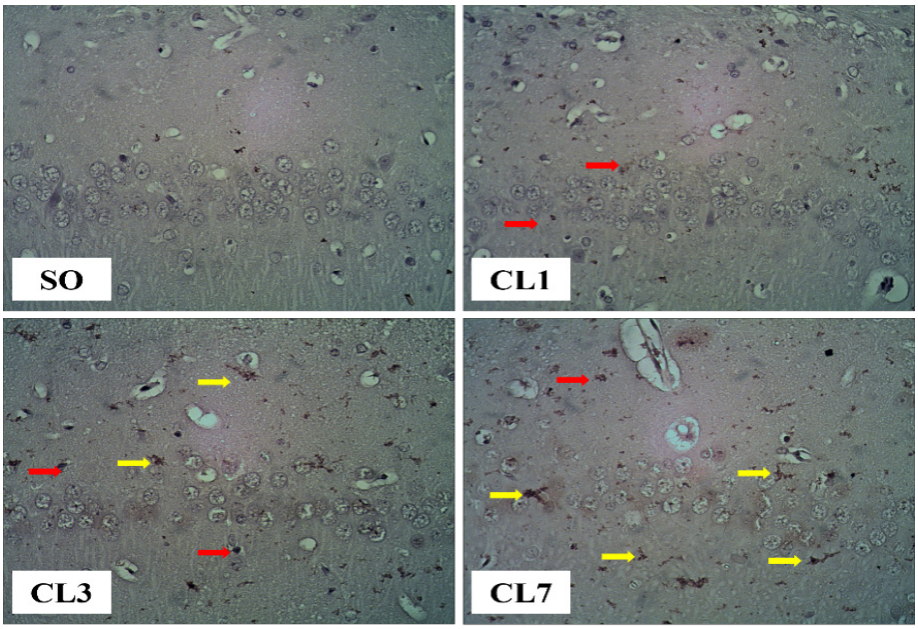

FIGURE 2. Immunohistochemical staining of CD68. Microscopic figures of CD68 positive fraction area. CD68 was found in the control and all ischemic groups. The activity of microglia is shown in the form of ameboid cells (red arrow) and the appearance of cell processes (yellow arrow).

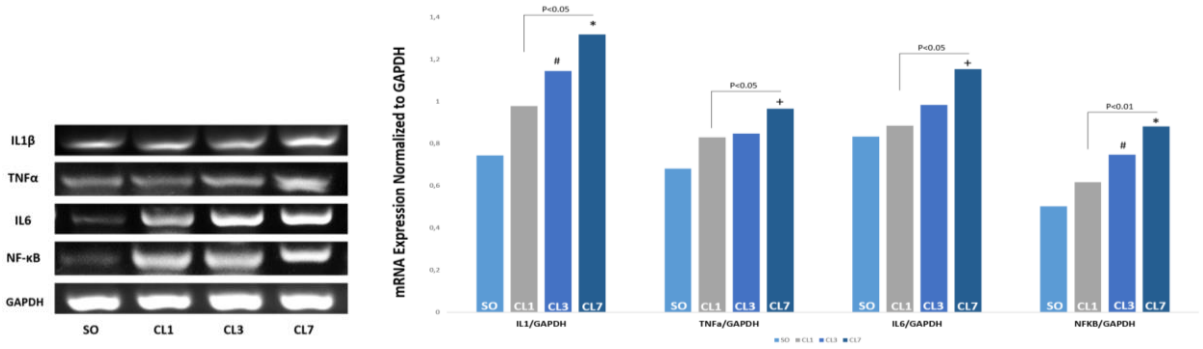

FIGURE 3. Densitometry analysis of mRNA expression of IL1β, IL6, TNFα, NF-κB, and GAPDH band using electrophoresis. Higher mRNA was observed in the CL1, CL3, and CL7 groups, compared to the SO. #: p<0.05 +: p<0.01 *: p<0.001 compared to SO

Correlation test

Statistical analysis using the Pearson correlation test showed strong positive correlation between NF-κB expression with CD68-positive fraction area (p=0.001; r=+0.616), TNFα expression (p=0.000; r=+0.765), IL1β expression (p=0.002; r=+0.601), and IL6 expression (p=0.000; r=+0.762).

DISCUSSION

Ischemic brain injury is a state in which a brain tissue's oxygen demand exceeds its supply of oxygen. Ischemia

of the brain might occur in people as a result of embolus formation or brain obstructions, which were reproduced in this work by ligating the CCA in rats. It is well known that brain hypoxia can induce inflammation.[18] The initial mechanism that occurs in this inflammatory process includes increased vascular permeability, the entry of leukocytes, increased temperature, and microglial activation.[19]

Microglia are the major resident immune cells in the brain that play an important role in generating the natural immune response.[20] As the inflammation response begins, microglia are activated and develop macrophage-like capabilities, including phagocytosis, secretion of cytokines, and release of MMP that leads to damage in the blood-brain barrier.[21] The activation of microglia due to ischemic state has dual effects, it can release many inflammatory mediators, which can be cytoprotective or cytotoxic.[22] In this study, the activation of microglia can be seen in the acute phase, and becoming more activated in the subacute and chronic phase as the ischemic damage worsens and the inflammatory response becomes more dominant. We can see the highest percentage of brown stained fraction area from CD68 immunopositivity in the chronic phase (CL7), followed by the subacute (CL3) and acute phase (CL1) respectively (FIGURE 1). The more active state of microglia also can be seen from the morphology of its amoeboid shape, found more abundant in chronic phase (FIGURE 2). Microglia can change its "normal" form, which is ramified, into hypertrophic, "bushy" form.[23] Microglia can also change their shape into amoeboid form in case severe tissue damage occurs or with pathogen invasion, primarily acting as phagocytic/macrophage cells and being difficult to distinguish from infiltrating macrophages.[23]

Microglia has 2 phenotypes after its activation, the M1 and M2. The process by which microglia turn into one of its phenotypes is called polarization. M1 phenotype has pro-inflammatory properties, while M2 is anti-inflammatory.[24] M1 will produce inflammatory mediators such as IL1β, IL6, and TNFα, while M2 produce anti-inflammatory cytokines such as IL4 and IL10. The present study observes that proinflammatory cytokines (IL1β, IL6, and TNFα) mRNA were upregulated and steadily increasing from the acute to chronic phase (FIGURE 3) suggesting most microglia underwent M1 phenotype differentiation.

Polarization of microglia into M1 phenotype in ischemic conditions is regulated by four types of modulators: transcription factors, ion channels, receptors, and gene modulators.[25] Knowing the regulator of microglia polarization is important because ischemic brain injury may be effectively treated by modulating this process. In this study, we investigate the role of NF-κB in microglial polarization. In the acute phase, NF-κB was increased compared to its normal condition (SO group), and toward the subacute and chronic phase, its level was much higher (FIGURE.3). Moreover, NF-κB has strong positive correlation with CD68 immunopositivity, and expression of IL6, IL1β, and TNFα suggesting NF-κB as a transcription factor initiating the polarization of microglia into M1 phenotype that produces proinflammatory mediator such as IL1β, IL6, and TNFα.

NF-κB, a family of transcription factors, is activated in response to harmful stresses and causes inflammatory reactions in the brain (26). Transcription factors included in it are RelA, RelB, cRel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52).[20] These transcription factors are attached in the cytoplasm by a class of inhibitors known as Inhibitors of κB (IκBs). Various mechanisms have been revealed that result in the nuclear accumulation and DNA binding of

NF-κB upon stimulation with various stressors. Canonical, non-canonical, and atypical pathways are subsets of these pathways. The canonical or classical method of NF-κB activation is the most well-characterized process, it entails activation of Transforming Growth Factor-B activating kinase (TAK1) and the Inhibitor of B kinase complex (IKK), which is constituted of IKKα, IKKβ, and IKKγ or NF-κB essential modulator (Nemo).[19,21]

Hypoxic conditions can trigger activation of the NF-kB system through several mechanisms such as dependent IKK mechanisms, the canonical signaling cascade, the role of prolyl-hydroxylases (PHDs), and the HIF inhibiting factor (FIH). Furthermore, inhibition of PHD will trigger activation of NF-kB, the role of Transforming Growth Factor Activating Kinase 1 (TAK1), and modulation of IκB.[19] This mechanism might explain the increased level of NF-kB mRNA expression in rats that underwent bilateral CCA ligation. Additionally, as demonstrated in previous literature, there is a correlation between NF-kB and macrophage polarization to type I macrophages.[15] NF-kB is a critical transcription factor for M1 activation due to multiple pathways that lead to M1 macrophage polarization.[15] NF-kB regulates the expression of several inflammatory genes such as TNFα, IL1β, cyclooxygenase 2 (COX2), IL6, and IL12p40.[22]

NF-κB activity is controlled by the inhibitor of kappa B kinase (IKK) trimeric complex, which consists of two kinases, IKKα, and IKKβ, and a regulatory protein, IKKγ (21). IKKβ is phosphorylated when upstream signals converge on the IKK complex, and IKKβ phosphorylates the inhibitory molecule inhibitor of kappa B (I-κB).[23] As a result, the NF-κB p65/p50 heterodimer is released from the NF-κB/I-κB complex, and I-κB is broken down by the proteasome.[22] The NF-κB p65/p50 heterodimer attached to the promoters of inflammatory genes after it translocated to the nucleus.[23] The TRIF adaptor pathway activates the transcription factor interferon-responsive factor 3 (IRF3) which results in the production and secretion of type I interferons, including IFNα and IFNβ.[24] This type of interferon binds to the type I interferon receptor (IFNAR) and then activates the transcription factor STAT1. IRF3, along with IRF5 was known to have roles in the induction of M1-associated gene and M1 polarization regulation.[25] The chemokines CXCL9 and CXCL10 were among the genes activated by IFN and showed classical M1 macrophage activation.[22] Indeed, macrophage polarization is intimately associated with the variable expression of many TLRs on the macrophage.[26]

Microglia become primed in situ when they are activated by proinflammatory cytokines such as IFNγ, IL1β, IL6, and TNFα.[27] Microglia can change its “normal” form, which is ramified, into a hypertrophic, “bushy” form.[28] Microglia can also change their shape into amoeboid form in case severe tissue damage occurs or with pathogen invasion, primarily acting as phagocytic/macrophage cells and being difficult to distinguish from infiltrating macrophages.[28] The proinflammatory M1 phenotype induces the secretion of pro-inflammatory cytokines that can worsen neural injury.[29]

Activation of M1 was indicated by an increase in the density of the area stained by CD68 as a marker of M1 which was significantly different in the carotid artery ligated group of rats compared to the control group. Several articles reported that mRNA expression level was increased due to brain ischemia. Kumar *et al.*,[30] described an increase in mRNA for IL-1, IL-12, TNFα, and iNOS in M1. Moreover, Morganti *et al.*,[31] saw an increase in mRNA expression for TNFα, IL1β, IL6, and IFNγ in M1. A study conducted by Braun also showed

an increase in mRNA for TNFα, and IL-12.[32] Lastly, an increased level of mRNA expression for TNFα, IL1β, IL6, and IFNγ was also depicted in a previous study.[33] Those findings might emphasize the results of our study, where in the case of acute and chronic brain ischemia modeled by artery ligation, there is an increase in IL1β, IL6, and TNFα mRNA. Such increment might be responsible for the brain damage observed in this case in the hippocampal area of CA1 and might be the cause of temporal lobe epilepsy.

CONCLUSION

In conclusion, NF-κB is the key player in hippocampal injury in the CA1 region following ischemic event by differentiating microglia into M1 phenotype form and initiates the neuroinflammatory cascade via IL1β, IL6, and TNFα in all phases.

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